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(54) Title: SITE-SPECIFIC HOMOGENEOUS MODIFICATION OF POLYPEPTIDES TO FACILITATE COVALENT LINKAGES TO A HYDROPHILIC MOIETY (57) Abstract A homogeneously modified protein is provided having one or more selected naturally occurring lysine residues replaced by a suitable amino acid, or having one or more lysine residues substituted for other amino acids or inserted into a polypeptide sequence, leaving selected lysine residues having ε-amino groups in the protein and coupling amine reactive compounds to selected lysine residues. Methods for producing the selected homogeneously modified proteins and pharmaceutical compositions containing such proteins are provided.		

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SITE-SPECIFIC HOMOGENOUS MODIFICATION OF POLYPEPTIDES
TO FACILITATE COVALENT LINKAGES TO A HYDROPHILIC MOIETY

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The present invention relates generally to polypeptides modified by the attachment thereto of compounds having amine reactive groups, methods for producing such modified polypeptides and compositions containing the modified polypeptides. More particularly, the invention relates to homogeneous modified polypeptides which are modified by attachment of hydrophilic moieties, including polymers, to selected positions in the polypeptide.

15

BACKGROUND

The desirability of modifying biologically active and therapeutically useful polypeptides with a variety of compounds having amine reactive groups, such as hydrophilic polymers, e.g., polyethylene glycol (PEG), to enhance their pharmacokinetic properties has been noted. See, e.g., the discussion of the art in this area of polypeptide modification in published PCT patent application WO87/00056. Such modification has been attempted to reduce adverse immune response to the polypeptide, increase the solubility for use in pharmaceutical preparations, and/or maintain a desirable circulatory level of such polypeptide for therapeutic efficacy.

One significant problem not addressed by the extensive art in this area of polypeptide modification involves the extent to which a polypeptide can be modified by attachment of compounds having amine reactive groups. For example, treatment of a polypeptide with PEG or similar polymers, can result in random attachment of the polymer at the amino terminus of the polypeptide and/or at one or more lysine residues in the amino acid sequence of the protein. While several PEG groups can attach to the polypeptide, the end result is a composition containing or potentially containing a variety of species of "PEG-ylated" polypeptide. Such heterogeneity in composition is undesirable for pharmaceutical use.

The attachment of compounds with amine reactive groups to a polypeptide may alter the biological activity of the polypeptide. This effect is believed mediated by the position and number of the attachment site(s) along the polypeptide sequence. There
5 thus remains in the art a need for a method enabling site specific attachment of such compounds to polypeptides, in a manner that enables the manipulation of the number and position of attachment sites. Such site specific attachments can generate homogeneously modified polypeptides which are therapeutically
10 efficacious and which retain certain desirable characteristics of the natural polypeptides.

Summary of the Invention

This invention provides materials and methods for site
15 specific covalent modification of polypeptides permitting the production of compositions comprising homogeneously modified polypeptides or proteins and pharmaceutical compositions containing same. "Homogeneously modified" as the term is used herein means substantially consistently modified only at specific
20 lysine residues. A homogeneously modified G-CSF, for example, includes a G-CSF composition which is substantially consistently modified at position 40, but not at positions 16, 23 and 34.

To solve the problem of non-specific susceptibility of polypeptides to covalent modification by amine-reactive moieties,
25 this invention first provides lysine-depleted variants ("LDVs") of polypeptides of interest. LDVs of this invention encompass polypeptides and proteins which contain fewer reactive lysine residues than the corresponding naturally occurring or previously known polypeptides or proteins. The lysine residues in the
30 peptide structure of the LDVs may occur at one or more amino acid positions occupied by lysine residues in the natural or previously known counterpart, or may be located at positions occupied by different amino acids in the parental counterpart. Furthermore, LDVs may in certain cases contain more lysine
35 residues than the parental counterpart, so long as the number of

lysine residues in the LDV permits homogeneous modification by reaction of the LDVs with amine-reactive moieties, as discussed below. Since such polypeptides or proteins of this invention contain a small number of lysine residues, generally six or less, preferably 1--4 lysines, they are also referred to herein as "LDVs" even though containing more lysine residues than the parental counterpart.

Polypeptides of interest include both proteins and polypeptides, preferably human, useful in therapeutic, prophylactic and/or diagnostic applications, including hematopoietins such as colony stimulating factors, e.g. G-CSF, GM-CSF, M-CSF, CSF-1, Meg-CSF, erythropoietin (EPO), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor, erythroid potentiating activity (EPA), macrophage activating factor, HILDA, interferons and tumor necrosis factor, among others; thrombolytic agents such as tPA, urokinase (uPA) and streptokinase and variants thereof as are known in the art; proteins involved in coagulation and hemostasis, including Factor V, Factor VII, Factor VIII, Factor IX, Factor XIII, Protein C and Protein S; proteins and polypeptides useful as vaccines; as well as other proteins and polypeptides and analogs thereof, including for example superoxide dismutase (SOD) (including extracellular SOD); growth hormones such as human and bovine growth hormone, epidermal growth factor, fibroblast growth factors, transforming growth factors $TGF\alpha$ and $TGF\beta$, insulin-like growth factor, PDGF, and ODGF; pulmonary surfactant proteins (PSPs); calcitonin; somatostatin; catalase; elastase; inhibins; angiogenic factors; atrial natriuretic factor; FSH, LH, FSH-releasing hormone, LH-releasing hormone and HCG; immunotoxins and immunoconjugates; anti-thrombin III; bone or cartilage morphogenic factors; and CD-4 proteins. In order to provide additional disclosure concerning exemplary proteins mentioned above and their uses, the following published foreign applications and co-owned pending U.S. applications are hereby incorporated by reference herein: PCT

Nos. WO 86/00639 and WO 85/05124; and U.S. Serial Nos. 940,362; 047,957; 021,865; and 099,938. Sequence information for other proteins mentioned above are also known in the art.

Most proteins and polypeptides contain several lysine residues within their peptide structure. By "lysine depleted variant" as the term is used herein, I mean variants of proteins or polypeptides which are modified in amino acid structure relative to naturally occurring or previously known counterparts in one or more of the following respects:

(i) at least one lysine residue of the natural or previously known compound is deleted or replaced with a substitute amino acid, preferably arginine;

(ii) at least one lysine residue is inserted into the natural or previously known sequence and/or is used to replace a different amino acid within that sequence; and,

(iii) the first amino acid at the N-terminus of the mature polypeptide is preferably proline, which is a relatively non-reactive amine, or is reversibly blocked with a protecting group.

With respect to modification (i), above, it is typically preferred in the case of lymphokines and other proteins of like molecular size that all but 1--6 of the original lysines be deleted and/or replaced. In general, for consistent homogeneous modification of the LDVs the fewer lysines remaining in the LDV the better, e.g. only 1--4 lysines. It should be understood, however, that in certain cases LDVs containing more than 4--6 reactive lysines may, given appropriate location and spacing of such lysines, be capable of homogeneous modification, e.g. PEGylation, and upon such modification may possess advantageous biological properties such as differential binding to receptors, antibodies or inhibitors relative to the parental protein, as discussed below. It should also be understood that in accordance

with modification (ii), above, one or more additional lysine residues may be inserted into the natural or previously known sequence and/or used to replace as desired other amino acids therein, e.g. arginine. Thus all lysines may be deleted or replaced in accordance with (i), and one or more new lysines may be inserted or used to replace a different amino acid in the molecule. Alternatively, all but one or two, for example, of the lysines in the natural or previously known sequence may be deleted or replaced with other amino acids, e.g. arginine. In any event, and as described in greater detail below, the LDVs of this invention make it possible for the first time to produce homogeneous compositions containing polypeptides or proteins (LDVs) substantially specifically and consistently modified at selected positions using amine-reactive moieties (described hereinafter) as the modifying agents.

Thus, in the practice of this invention, lysine residues are identified in those portions of the polypeptide where modification via amino-reactive moieties is not desired. The lysine residues so identified are deleted or replaced with different amino acids, e.g. by genetic engineering methods as described below. Preferably replacements are conservative, i.e. lysine is replaced by arginine, and where a new lysine is to be introduced, arginine by lysine. Any remaining lysine residues represent sites where modification by amine-reactive moieties is desired. Alternatively, or in addition, novel lysine residues may be engineered into the polypeptide at positions where attachment is desired, most conveniently, for example, by simple insertion of a lysine codon into the DNA molecule at the desired site or by converting a desirably located arginine or other codon to a lysine codon. Convenient methods for (i) site specific mutagenesis or DNA synthesis for producing a DNA molecule encoding the desired LDV, (ii) expression in procaryotic or eucaryotic host cells of the DNA molecule so produced, and (iii) recovery of the LDV produced by such expression are also disclosed in detail below.

The LDVs of this invention retain useful biological properties of the natural or previously known polypeptide or protein, and may thus be used, with or without modification with amine-reactive moieties, for applications identified for the non-
5 modified parent polypeptide or protein. Modification with such moieties, however, is preferred. Such modified LDVs are producable in homogeneous compositions which, it is contemplated, will provide improved pharmacokinetic profiles and/or solubility characteristics relative to the parent polypeptides.

10 In cases where the parental polypeptide normally can interact with one or more receptors, as in the case of IL-2 for example, it is contemplated that modified LDVs of the polypeptide wherein the modification masks one or more receptor binding sites may interact e.g. with only one type of its receptors, i.e. not
15 interact with one or more other types of receptors which interact with the parental polypeptide. Such modified LDVs may represent therapeutic agents having more specific biological and pharmacologic activities than the corresponding parental polypeptide. In cases where the parental polypeptide normally can interact with
20 an inhibitor, as in the case of tPA, it is contemplated that modified LDVs of such polypeptides or proteins wherein the modification masks an inhibitor binding site may have a reduced or substantially abolished interaction with the inhibitor, and thus improved utility as a therapeutic agent. In cases where the
25 natural or recombinant protein can elicit neutralizing or otherwise inhibitory antibodies in humans, as in the case of Factor VIII, modified LDVs wherein the modification masks the epitope for such antibodies may represent the first potential therapeutic, and indeed, life saving, agents. Finally, where
30 specific proteolytic cleavage inactivates or otherwise negatively affects therapeutic utility of a protein, as in the case of the APC cleavage site in Factor VIII or the proteolytic cleavage site in prourokinase which liberates the kringle region from the serine protease domain, modified LDVs of the protein wherein the
35 modification masks the cleavage site may represent potential

therapeutic agents with longer effective in vivo half life or other improved properties relative to the parental protein.

Biological activity of the LDVs before or after modification with the amine-reactive moieties may be determined by standard in vitro or in vivo assays conventional for measuring activity of the parent polypeptide.

Selective and homogeneous modification of the LDVs with amine-reactive moieties is possible since such moieties will covalently bond only to ϵ -amino groups of the remaining lysine residue(s) in the LDVs and to the amino terminus of the LDV, if reactive. The modified LDVs so produced may then be recovered, and if desired, further purified and formulated with into pharmaceutical compositions by conventional methods.

It is contemplated that certain polypeptides or proteins may contain one or more lysine residues, which by virtue of peptide folding or glycosylation, for example, are not accesible to reaction with amine-reactive moieties, except under denaturing conditions. In the practice of this invention such non-reactive lysine residues may be, but need not be, altered since they will not normally be susceptible to non-specific modification by amine-reactive moieties. The presence in parental polypeptides or proteins of non-reactive lysine residues may be conveniently determined, if desired, by modifying the parental polypeptide or protein with an amine-reactive compound which results in the attachment to reactive lysines of a modifying moiety of known molecular weight under denaturing and non-denaturing conditions, respectively, and determining, e.g. by SDS-PAGE analysis, the number of attached moieties in each case. The presence and number of additional attached moieties on the denatured parental polypeptide relative to the non-denatured parental polypeptide is a general indication of the presence and number of non-reactive lysine residues. The locations of any such non-reactive lysine residues may be determined, e.g. by SDS-PAGE analysis of proteolytic fragments of the polypeptide modified under denaturing and non-denaturing conditions. Lysine residues which

Amine-reactive moieties include compounds such as succinic anhydride and polyalkylene glycols, e.g. polyethylene and polypropylene glycols, as well as derivatives thereof, with or without coupling agents or derivatization with coupling moieties, e.g. as disclosed in U.S. Patent No. 4,179,337; published European Patent Application No. 0 154 316; published International Application No. WO 87/00056; and Abuchowski and Davis, in "Enzymes as Drugs" (1981), Hokenberg & Roberts, eds. (John Wiley & Sons, NY), pp. 367-383.

$$\text{---(Lys)}_n\text{---} + \geq n(\text{Y-Z}) \longrightarrow \text{---(Lys)}_n\text{---}$$

|
Y

Briefly, the method comprises reacting the LDV with an amine reactive compound under suitable conditions, preferably non-denaturing conditions, and in sufficient amounts permitting the covalent attachment of the hydrophilic moiety to lysine residue(s) present in the polypeptide backbone of the LDV. Generally, the amount of amine-reactive compound used should be at least equimolar to the number of lysines to be derivatized, although use of excess amine-reactive compound is strongly preferred, both to improve the rate of reaction and to insure consistent modification at all reactive sites. The modified LDV so produced, may then be recovered, purified and formulated by

conventional methods. See e.g., WO 87/00056 and references cited therein

While any polypeptide is a candidate for the method of the invention, presently desirable polypeptides to be homogeneously modified include lymphokines and growth factors. Of significant interest are those polypeptides which affect the immune system, including the colony stimulating factors, and other growth factors.

Other aspects of the present invention include therapeutic methods of treatment and therapeutic compositions which employ the modified polypeptide LDVs of the present invention. These methods and compositions take advantage of the improved pharmacokinetic properties of these modified LDVs to provide treatments, e.g., such as employing lower dosages of polypeptide, less frequent administration, and more desirable distribution, required for the therapeutic indications for the natural polypeptide.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of the invention, including illustrative examples of the practice thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the polypeptide sequence of IL-2, with amino acid numbers used for reference in the specification.
Fig. 2 is the polypeptide sequence of IL-3, with amino acid numbers used for reference in the specification.
Fig. 3 is the polypeptide sequence of IL-6, with amino acid numbers used for reference in the specification.
Fig. 4 is the polypeptide sequence of G-CSF, with amino acid numbers used for reference in the specification.
Fig. 5 illustrates synthetic oligonucleotides for the preparation of synthetic DNA molecules encoding exemplary IL-2 LDVs of the invention; odd numbered oligonucleotides correspond to sequences

within sense strands, even numbered oligonucleotides to anti-sense strands; the initiation ATG is marked with "****" and altered codons are underlined; oligonucleotides in Fig. 5A yield the LDV with alanine at position 125 and oligonucleotides in Fig. 5B yield the LDV with cysteine at position 125.

DETAILED DESCRIPTION OF THE INVENTION

The present invention involves the selective modification of polypeptides of interest for pharmaceutical use, to both enhance their pharmacokinetic properties and provide homogeneous compositions for human therapeutic use. Any polypeptide is susceptible to use in the method of the invention. Most desirably, a polypeptide having one or more lysine residues in its amino acid sequence, where it would be desirable to attach an amine reactive compound, may be employed. Also polypeptides having arginine residues which may be converted to lysine residues for such attachments may be employed. Lysine residues may also or alternatively be inserted into, or used to replace endogenous amino acid residues, in a polypeptide a sequence which has no conveniently located lysine or arginine residues. Finally, lysine residues may be used to replace asparagine, serine or threonine residues in consensus N-linked glycosylation sites. In the latter case, the LDVs, even when expressed in bacterial cells (and refolded if necessary or desired), may be derivatized as disclosed herein at one or more locations otherwisely glycosylated when expressed in eukaryotic cells.

The method for selectively modifying the polypeptide of choice involves selecting locations in the polypeptide sequence for the attachment of amine reactive compounds. This step may be accomplished by altering the amino acid sequence of the polypeptide by converting selected lysine residues into arginine residues, or converting selected arginine residues into lysine residues. For example, the codons AAA or AAG, which code for lysine, can be changed to the codons AGA, AGG, CGA, CGT, CGC, or CGG which code for arginine, and vice versa. Alternatively,

lysine residues may be inserted into and/or deleted from a peptide sequence at a selected site(s).

LDVs in accordance with this invention also include proteins with allelic variations, i.e. sequence variations due to natural variability from individual to individual, or with other amino acid substitutions or deletions which still retain desirable biological properties of the parental protein or polypeptide.

All LDVs of this invention may be prepared by expressing recombinant DNA sequences encoding the desired variant in host cells, e.g. procaryotic host cells such as *E. coli*, or eucaryotic host cells such as yeast or mammalian host cells, using methods and materials, e.g. vectors, as are known in the art. DNA sequences encoding the variants may be produced synthetically or by conventional site-directed mutagenesis of DNA sequences encoding the protein or polypeptide of interest or analogs thereof.

DNA sequences encoding various proteins of interest have been cloned and the DNA sequences published. DNA sequences encoding certain proteins of interest have been deposited with the American Type Culture Collection (See Table 1). DNA molecules encoding a protein of interest may be obtained (i) by cloning in accordance with published methods, (ii) from deposited plasmids, or (iii) by synthesis, e.g. using overlapping synthetic oligonucleotides based on published sequences which together span the desired coding region.

As mentioned above, DNA sequences encoding individual LDVs of this invention may be produced synthetically or by conventional site-directed mutagenesis of a DNA sequence encoding the parental protein or polypeptide of interest or analogs thereof. Such methods of mutagenesis include the M13 system of Zoller and Smith, *Nucleic Acids Res.* 10:6487 - 6500 (1982); *Methods Enzymol.* 100:468-500 (1983); and *DNA* 3:479-488 (1984), using single stranded DNA and the method of Morinaga et al., *Bio/technology*, 636-639 (July 1984), using heteroduplexed DNA. Exemplary oligonucleotides used in accordance with such methods are described below. It should be understood, of course, that

DNA encoding each of the LDVs of this invention may be analogously produced by one skilled in the art through site-directed mutagenesis using appropriately chosen oligonucleotides.

5 The new DNA sequences encoding the LDVs of this invention can be introduced into appropriate vectors for heterologous expression in the desired host cells, whether procaryotic or eucaryotic. The activity produced by the transiently transfected or stably transformed host cells may be measured by using
10 standard assays conventional for the parental protein.

 The LDV produced by expression in the genetically engineered host cells may then be purified, and if desired formulated into pharmaceutical compositions by conventional methods, often preferably by methods which are typically used in purifying
15 and/or formulating the parental protein. It is contemplated that such pharmaceutical compositions containing the LDV in admixture with a pharmaceutically acceptable carrier will possess similar utilities to those of the parental proteins.

 In another, and preferred, aspect of this invention, the
20 LDVs produced by recombinant means as mentioned above are reacted with the desired amine-reactive compound under conditions permitting attachment of the compound to the ϵ -amino groups at remaining lysine residues in the peptide backbone of the LDV.

 The term "amine reactive compound" is defined herein as any
25 compound having a reactive group capable of forming a covalent attachment to the Epsilon amine group of a lysine residue. Included among such compounds are hydrophilic polymers such as PEG and polypropylene glycol (PPG); compounds such as succinic anhydride; and others. Methods for such attachment are
30 conventional, such as described in PCT application WO97/00056 and references described therein. However, by controlling the number and location of the remaining lysines in the LDV sequence, the number and location(s) of the attached moiety can be selectively controlled. Such control of attachment location and number
35 enables the production of only certain selectively modified

molecules retaining the desired biological activity, rather than production of a heterogeneous mixture of variably modified molecules, only some of which may be active.

Another aspect of the invention is therefore homogeneous compositions of modified LDVs as described herein, e.g. PEGylated LDVs. Specific embodiments of polypeptide LDVs of the invention include IL-2 which has arginine residues replacing lysine residues at one or more of the lysine residues at positions 8, 9, 32, 35, 43, 48, 49, 54, 64, 76, and 97. A presently desirable example of such a modified IL-2 has the natural lysine residue only at position 76, with all other lysine residue positions as identified above being replaced by arginine residues and with lysine 76 being coupled to PEG. Amino acid numbers correlate with the numbering system used in Fig. 1 for the appropriate unmodified peptides.

Similarly, one or more of the naturally occurring lysine residues in IL-3 (Fig. 2) at amino acid positions 10, 28, 66, 79, 100, 110 and 116 may be converted to a suitable amino acid, such as arginine, to create a polypeptide LDV of the invention. For example, one such polypeptide has positions 10, 28, 100, 110 and 116 converted to arginine and the remaining lysine residues at positions at 79 and 66 coupled to PPG. Alternatively one or more of the arginine residues may be converted to lysine residues. Table 2 below illustrates the positions and amino acid numbers of lysine and arginine residues in several exemplary polypeptides which can be altered according to the invention. The position numbers correspond to the appropriate figures 1 through 4. In the case of EPO, it may be desirable to replace all but one to about four of the endogenous lysine residues (positions 20, 45, 52, 97, 116, 140, 152 and 154) with arginine residues and/or to convert one or more of the endogenous arginine residues to lysine residues, especially at positions 4 and/or 10 and/or 162.

Other modified peptides may be selected and produced in accordance with this invention as described for the above peptides, which are included as examples only.

Table 1: DNA encoding exemplary proteins of interest

	<u>protein</u>	<u>vector & ATCC accession #</u>	<u>references</u>
5	G-CSF	pxMT2G-CSF (67514)	(1)
	GM-CSF	pCSF-1 (39754)	(2)
	M-CSF	p3ACSF-69 (67092)	(3)
	CSF-1		(4)
	IL-2	pBR322-aTCGF (39673)	(6)
10	IL-3	pCSF-MLA (67154); CSF-16 (40246); pHuIL3-2 (67319); pSHIL-3-1 (67326)	(7)
	IL-6	pCSF309 (67153); pAL181(40134)	(8)
	tPA	pIVPA/1 (39891); J205 (39568)	(9)
	FVIII	pSP64-VIII (39812); pDGR-2(53100)	(10)
15	ATIII	p91023 AT III-C3 (39941)	(11)
	SOD		(12)
	EPO	RKFL13 (39989)	(13)
20	1. US Serial No. 099,938 and references cited therein; published PCT WO 87/01132.		
	2. WO 86/00639; Wong et al., Science		
	3. WO 87/06954		
	4. Kawasaki et al., 1985, Science <u>230</u> :291-296		
	6. US Serial No. 849,234 (filed April 6, 1986)		
25	7. PCT/US87/01702		
	8. PCT/US87/01611		
	9. WO 87/04722; US Serial Nos. 861,699; 853,781; 825,104; and 882, 051; US Serial No. 566,057; D. Pennica et al., 1983, Nature <u>301</u> :214; Kaufman et al., 1985, Mol. Cell. Biol. 5(7):1750 et seq.		
30	10. GI 5002; WO 87/07144		
	11. US Serial Nos. 677,813; 726,346; and 108,878; US Patent No. 4,632,981		
	12. WO 87/01387		
35	13. WO 86/03520		

Amine-reactive compounds will typically also react with the amino terminus of a polypeptide under the conditions described above, so long as the amino terminus is accessible to amine-reactive agents (i.e. reactive) and is not blocked. Therefore an alternatively modified polypeptide may be provided by blocking the reactive site on the amino terminus of the selected polypeptide LDV before reacting the LDV with the desired amine-reactive compound. Unblocking the N-terminus after the modifying moiety, e.g. polymer, has been covalently linked to LDV lysines will produce a modified polypeptide with polymer or other modifying moiety attached to the remaining lysines in the amino acid sequence of the LDV, but not at the amino terminus. Thus, compositions of polypeptides homogeneous for polymer attachment or lack of polymer attachment at the amino terminus are also encompassed by this invention. Additionally, for bacterial expression where the secretory leader-encoding DNA sequence is removed from the LDV-encoding DNA, it may be desirable to additionally modify the sequence such that it encodes an N-terminus comprising Met-Pro--- instead of other N-termini such as Met-Ala-Pro. Such N-terminal modification permits more consistent removal of the N-terminal methionine.

Thus, LDVs of this invention, modified as described, encompass LDVs containing other modifications as well, including truncation of the peptide sequence, deletion or replacement of other amino acids, insertion of new N-linked glycosylation sites, abolishment of natural N-linked glycosylation sites, etc. Thus, this invention encompasses LDVs encoded for by DNA molecules which are capable of hybridizing under stringent conditions to the DNA molecule encoding the parental polypeptide or protein so long as one or more lysine residues of the parental peptide sequence is deleted or replaced with a different amino acid and/or one or more lysine residues are inserted into the parental peptide sequence and the resulting LDV is covalently modified as described herein.

Because the method and compositions of this invention provide homogeneous modified polypeptides, the invention also encompasses such homogeneous compositions for pharmaceutical use which comprise a therapeutically effective amount of a modified LDV described above in a mixture with a pharmaceutically acceptable carrier. Such composition can be used in the same manner as that described for the natural or recombinant polypeptides. It is contemplated that the compositions will be used for treating a variety of conditions. For example, a modified IL-2 may be used to treat various cancers. A modified G-CSF can be used to treat neutropenia, e.g., associated with chemotherapy. A modified EPO may be used for treating various anemias. The exact dosage and method of administration will be determined by the attending physician depending on the particular modified polypeptide employed, the potency and pharmacokinetic profile of the particular compound as well as on various factors which modify the actions of drugs, for example, body weight, sex, diet, time of administration, drug combination, reaction sensitivities and severity of the particular case. Generally, the daily regimen should be in the range of the dosage for the natural or recombinant unmodified polypeptide, e.g. for a colony stimulating factor such as G-CSF, a range of 1-100 micrograms of polypeptide per kilogram of body weight.

TABLE 2

<u>IL-2 residues</u>		<u>IL-6 residues</u>	
lysine	arginine	lysine	arginine
5			
8	38	10	17
9	81	28	25
32	83	42	31
35	120	47	41
10		55	105
43		67	114
48		71	169
49		87	180
54		121	183
64		129	
15		130	
76		132	
97		151	
		172	
20			
<u>G-CSF residues</u>		<u>IL-3 residues</u>	
lysine	arginine	lysine	arginine
16	22	10	54
23	146	28	55
25	147	66	63
34	166	79	94
40	169	100	108
		110	109
		116	
30			

The therapeutic method and compositions of the present invention may also include co-administration with other drugs or human factors. The dosage recited above would be adjusted to
5 compensate for such additional components in the therapeutic composition or regimen. In the case of pharmaceutical compositions containing modified lymphokine LDVs, for example, progress of the treated patient can be monitored by periodic assessment of the hematological profile, e.g. white cell count, hematocrit and the
10 like.

The following examples illustrate the method and compositions of the invention.

EXPERIMENTAL MATERIALS, METHODS AND EXAMPLES

15 Example 1: Eucaryotic Expression Materials and Methods

Eukaryotic cell expression vectors into which DNA sequences encoding LDVs of this invention may be inserted (with or without synthetic linkers, as required or desired) may be synthesized by techniques well known to those skilled in this art. The components of the vectors such as the bacterial replicons, selection
20 genes, enhancers, promoters, and the like may be obtained from natural sources or synthesized by known procedures. See Kaufman et al., J. Mol. Biol., 159:601-621 (1982); Kaufman, Proc. Natl. Acad. Sci. 82:689-693 (1985). See also WO 87/04187 (pMT2 and pMT2-ADA) and US Serial No. 88,188, filed August 21, 1987)(pXMT2). Exemplary vectors useful for mammalian expression are also disclosed in the patent applications cited in Example 4, which are hereby incorporated by reference. Eucaryotic expression vectors useful in producing variants of this invention
25 may also contain inducible promoters or comprise inducible expression systems as are known in the art. See US Serial No. 893,115 (filed August 1, 1986) and PCT/US87/01871.

Established cell lines, including transformed cell lines, are suitable as hosts. Normal diploid cells, cell strains
35 derived from in vitro culture of primary tissue, as well as

primary explants (including relatively undifferentiated cells such as haematopoietic stem cells) are also suitable. Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominantly acting.

5 The host cells preferably will be established mammalian cell lines. For stable integration of the vector DNA into chromosomal DNA, and for subsequent amplification of the integrated vector DNA, both by conventional methods, CHO (Chinese Hamster Ovary) cells are presently preferred. Alternatively, the vector DNA may
10 include all or part of the bovine papilloma virus genome (Lusky et al., Cell, 36: 391-401 (1984) and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other usable mammalian cell lines include HeLa, COS-1 monkey cells, melanoma cell lines such as Bowes cells, mouse L-929 cells, 3T3
15 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines and the like.

Stable transformants then are screened for expression of the LDV product by standard immunological or activity assays. The presence of the DNA encoding the LDV proteins may be detected by
20 standard procedures such as Southern blotting. Transient expression of the procoagulant genes during the several days after introduction of the expression vector DNA into suitable host cells such as COS-1 monkey cells is measured without selection by activity or immunologic assay of the proteins in the culture
25 medium.

Following the expression of the DNA by conventional means, the variants so produced may be recovered, purified, and/or characterized with respect to physiochemical, biochemical and/or clinical parameters, all by known methods.

30

Example 2: Bacterial and Yeast expression

Bacterial and yeast expression may be effected by inserting (with or without synthetic linkers, as required or desired) the DNA molecule encoding the desired LDV into a suitable vector (or
35 inserting the parental DNA sequence into the vector and mutagenizing the sequence as desired therein), then transforming

the host cells with the vector so produced using conventional vectors and methods as are known in the art, e.g. as disclosed in published PCT Application No. WO 86/00639. Transformants are identified by conventional methods and may be subcloned if
5 desired. Characterization of transformants and recombinant product so produced may be effected and the product recovered and purified, all as described in Example 1.

For bacterial expression, the DNA sequences encoding the LDVs are preferably modified by conventional procedures to encode
10 only the mature polypeptide and may optionally be modified to include preferred bacterial codons.

Where the LDV comprises lysine residues at one or more locations otherwise occupied in the native sequence by consensus N-linked glycosylation sites or by an O-linked glycosylation
15 site, modification (e.g. PEGylation) of the bacterial (or other) expression product (refolded if necessary or desired) results in a polypeptide more closely mimicing the corresponding native glycosylated eucaryotic expression product..

20 Example 3: Insect Cell Expression

Similarly, expression of the recombinant LDVs may be effected in insect cells, e.g. using the methods and materials disclosed therefor in published European Applications Nos. 0 155 476 A1 or 0 127 839 A2 and in Miller et al., Genetic
25 Engineering, Vol.8, pp.277-298 (J.K. Setlow and A. Hollander, eds., Plenum Press, 1986); Pennock et al., 1984, Mol. Cell. Biol. 4:(3)399-406; or Maeda et al., 1985, Nature 315:592-594.

Example 4: Mutagenesis Protocol

30 Site directed mutagenesis may be effected using conventional procedures known in the art. See e.g. published International Applications Nos. WO 87/07144 and WO 87/04722 and US Serial Nos. 099,938 (filed September 23, 1987) and 088,188 (filed August 21, 1987) and the references cited therein.

Example 5: Exemplary Oligonucleotides for Mutagenesis Reactions

The following oligonucleotides were designed for the indicated exemplary mutagenesis reactions:

5	#	sequence	modification
		[IL-2	K--->R at position:]
	1	CA AGT TCT ACA <u>AGG</u> AAA ACA CAG C	8
	2	GT TCT ACA AAG <u>AGA</u> ACA CAG CTA C	9
	3	GGA AAT AAT TAC <u>AGG</u> AAT CCC AAA C	32
10	4	C AAG AAT CCC <u>AGA</u> CTC ACC AGG ATG C	35
	5	G CTC ACA TTT <u>AGG</u> TTT TAC ATG CCC	43
	6	G TTT TAC ATG CCC <u>AGG</u> AAG GCC ACA GAA C	48
	7	G TTT TAC ATG CCC AAG <u>AGG</u> GCC ACA GAA C	49
	8	GCC ACA GAA CTG <u>AGA</u> CAT CTT CAG TG	54
15	9	GAA GAA GAA CTC <u>AGA</u> CCT CTG GAG G	64
	10	GCT CAA AGC <u>AGA</u> AAC TTT CAC TTA AG	76
	11	GTT CTG GAA CTA <u>AGG</u> GGA TAT GAA AC	97
		R--->K at position:	
	12	CCC AAA CTC ACC <u>AAG</u> ATG CTC ACA TTT	38
20	13	C TTT CAC TTA <u>AAA</u> CCC AGG GAC	81
	14	CAC TTA AGA CCC <u>AAG</u> GAC TTA ATC AGC	83
	15	GAA TTT CTG AAC <u>AAA</u> TGG ATT ACC TTT TG	120
		[G-CSF	K---->R at position:]
	16	GC TTC CTG CTC <u>AGG</u> TGC TTA GAG C	16
25	17	G CAA GTG AGG <u>AGG</u> ATC CAG GGC G	23
	18	GCG CTC CAG GAG <u>AGG</u> CTG TGT GCC ACC	34
	19	GT GCC ACC TAC <u>AGG</u> CTG TGC CAC CCC	40
		R---->K at position:]	
	20	GC TTA GAG CAA GTG <u>AAG</u> AAG ATC CAG GGC	22
30	21	CT GCT TTC CAG <u>AAA</u> CGG GCA GGA GGG	146
	22	GCT TTC CAG CGC <u>AAG</u> GCA GGA GGG GTC C	147
	23	GAG GTG TCG TAC <u>AAG</u> GTT CTA CGC CAC C	166
	24	C CGC GTT CTA <u>AAG</u> CAC CTT GCC CAG CCC	169

35 In the exemplary oligonucleotides depicted above regions designed to effect a codon alteration are underlined. It should

be understood of course that the depicted list of oligonucleotides is merely exemplary and not exclusive. The design and synthesis of alternative and additional oligonucleotides for mutagenesis in accord with this invention is well within the present skill in the art given the benefit of this disclosure.

Synthesis of such oligonucleotides may be conveniently effected using conventional automated DNA synthesis equipment and methods, typically following the manufacturer's instructions.

One skilled in the art, of course, could readily design and synthesize other oligonucleotides for deletion of lysine codons or insertion thereof in DNA sequences encoding IL-2 or G-CSF. Additionally, one could also readily design and synthesize other oligonucleotides for similar mutagenesis of DNA encoding any desired protein or polypeptide for use in the production of LDVs of this invention. To modify more than one site mutagenesis may be carried out iteratively, or in some cases using an oligonucleotide designed for mutagenesis at more than one site. For example, to modify a DNA molecule encoding IL-2 to encode R-48, R-49 IL-2 one may mutagenize the parental DNA molecule iteratively using oligonucleotides 6 and 7, depicted above. Alternatively, one could mutagenize with the following oligonucleotide:

G CTC ACA TTT AAG TTT TAC ATG CCC AGG AGG -
GCC ACA GAA CTG AAA CAT CTT CAG

which is designed to effect both mutagenesis reactions.

By way of example, one may readily produce a DNA molecule and express it to yield one of the following G-CSF LDVs:

Exemplary G-CSF LDVs

- | | | |
|----|---------------------|---|
| 30 | 1. R-16 G-CSF | 9. R-23, R-40 G-CSF |
| | 2. R-23 G-CSF | 10. R-34, R-40 G-CSF |
| | 3. R-34 G-CSF | 11. R-16, R-23, R-34 G-CSF |
| | 4. R-40 G-CSF | 12. R-16, R-34, R-40 G-CSF |
| | 5. R-16, R-23 G-CSF | 13. R-23, R-34, R-40 G-CSF |
| 35 | 6. R-16, R-34 G-CSF | 14. K-169, R-16, R-23, R-34, R-40 G-CSF |
| | 7. R-16, R-40 G-CSF | 15. R-16, R-34, K-147 G-CSF |
| | 8. R-23, R-34 G-CSF | |

Modification by methods described herein of such G-CSF LDVs, for example, provides the following exemplary modified G-CSF LDVs:

```

[-----K---R-K---K---K-----R-R---R-R----- ]
5  |-----R---R-R---R---R-----R-R---R-R-----
   |-----R---R---R---R-----R-R---R-R-----
10 |-----R---R---R---R-----R-R---R-R-----
   |-----R---R---R---R-----R-R---R-R-----
15 |-----R---R-R---|-----R-R---R-R-----
   |-----R---R---R---R-----|-----R-R-----
20 |-----R---R-R---R---R-----R-R---|-----R-----
   |-----R---R-R---R---R-----R-R---R---|-----
25 |-----R---R-R---R---R-----R-R---R---|-----
   |-----R---R-R---R---R-----R-R---|-----
30 |-----R---R-R---R---R-----R-R---R-R---|-----
   |-----R---R---|-----R---|-----R---R-----
35

```

wherein " | " represents a modification in accordance with this invention, e.g. PEGylation, at each reactive lysine residue in the LDV. The parental peptide sequence of G-CSF is depicted schematically at the top in brackets indicating the relative locations of positions 16, 23, 34 and 40 (occupied by lysine residues in G-CSF) and 22, 146, 147, 166 and 169 (occupied by arginine residues in G-CSF). As depicted schematically above, all lysines not intended as potential attachment sites were replaced with arginine. It should be understood of course, that

as previously mentioned, lysines not intended as potential attachment sites may be replaced with other amino acids as well, or simply deleted, and one or more additional lysine residues may be added by insertion between or replacement of amino acid of the parental peptide sequence.

Example 6: Synthesis of DNA molecules encoding LDVs

As an alternative to the production of LDV-encoding DNA by mutagenesis of a parental DNA sequence, it should be understood that the desired LDV-encoding DNA may be prepared synthetically. In that case, it will usually be desirable to synthesize the DNA in the form of overlapping oligonucleotides, e.g. overlapping 50-80mers, which together span the desired coding sequence:

_____ ...
 _____

Given a desired coding sequence, the design, synthesis, assembly and ligation, if desired, to synthetic linkers of appropriate oligonucleotides is well within the persent level of skill in the art.

Example 7: Preparation of PEG-ylated IL-2 LDV

a. DNA encoding the LDV

A DNA molecule encoding IL-2 containing arginine residues in place of lysine residues at positions 8, 9, 32, 35, 43, 48, 54, 64 and 97 (and alanine in place of cystein at position 125) is synthesized as follows. The oligonucleotides depicted in Fig. 5A are synthesized by conventional means using a commercial automated DNA synthesizer following the supplier's instructions. Odd numbered oligonucleotides in Fig. 5 are "sense" strands, even numbered oliognucleotides are "anti-sense" strands. Oligonucleotides 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14 and 15 and 16 are annealed to each other, respectively, under conventional conditions, e.g. 10mM tris, PH 7.5, 20mM NaCl,

2mM MgCl₂, and 10pM (combined oligonucleotides)/λ of solution, with heating to 100 C followed by slow cooling over ~2 h to 37 C. The eight mixtures are then combined and the duplexes were ligated to one another under standard conditions, e.g. 50mM tris, 5 pH 7.4, 10mM MgCl₂, 10mM DTT, and 1 mM ATP and 5 Weiss units of T4 ligase (New England BioLabs) at room temperature overnight (~16 h). The mixture is electrophoresed through a 1% low gelling temperature agarose gel and a band of 480 bp was excised from the gel. That DNA molecule so produced encodes an Ala-125 IL-2 10 having the K-->R mutations indicated above on an EcoRI/XhoI cassette.

b. insertion into vector, expression and modification of the LDV

The EcoRI/Xho I cassette may then be inserted into any 15 desired vector, e.g. pxMT2 or derivatives thereof, using synthetic linkers as desired or necessary. Transformation of mammalian cells, e.g. COS or CHO cells, selection of transformants, amplification of gene copy number in the case of CHO transformants, and culturing of the cells so obtained to 20 produce the desired LDV, may be readily effected by conventional methods, such as those disclosed in the references in Table 1, above. The protein so produced may be recovered and further purified if desired, and PEGylated, and the PEGylated protein purified all by conventional methods.

25

Example 8: Preparation of Alternative PEGylated IL-2 LDV

Example 8 may be repeated using the oligonucleotides depicted in Fig. 5B in place of those depicted in Fig. 5A. The DNA molecule so produced encodes an LDV identical to that in 30 Example 8, except that cystein at position 125 is retained. The corresponding PEGylated IL-2 LDV is thus produced.

Example 9: Preparation of PEG-ylated R-16, R-34, K-147 G-CSF LDV

pxMT2G-CSF may be mutagenized by conventional procedures 35 using oligonucleotides 16, 18 and 22 depicted in Example 5 to produce a pxMT2G-CSF derivative encoding the title G-CSF LDV.

Transformation of mammalian cells, e.g. COS or CHO cells, selection of transformants, amplification of gene copy number in the case of CHO transformants, and culturing of the cells so obtained to produce the desired LDV, may be readily effected by
5 conventional methods, such as those disclosed in the references in Table 1, above. The protein so produced may be recovered and further purified if desired, PEGylated by conventional procedures and the PEGylated protein purified by standard methods.

10

The same or similar procedures may be used by one skilled in the art to attach polymers such as PEG or PPG or other moieties, preferably hydrophilic moieties, to the other LDVs of the invention. Homogeneity can be observed by conventional analysis
15 of the modified LDVs so produced e.g. using standard SDS-PAGE or HPLC analysis.

20

Numerous modifications may be made by one skilled in the art to the methods and compositions of the present invention in view of the disclosure herein. Such modifications are believed to be encompassed by this invention as defined by the appended claims.

25

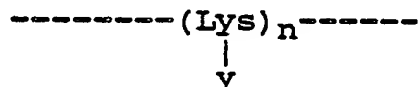
What is claimed is:

1. A lysine depleted variant ("LDV") of a lymphokine, growth factor, hormone or vaccination agent having biological activity characterized by the deletion of, or amino acid substitution for, at least one lysine residue; and/or the insertion of a lysine residue within the polypeptide sequence and/or the replacement of a different amino acid within the polypeptide sequence with a lysine residue.
2. An LDV of claim 1, wherein the amino acid substitution for lysine comprises the substitution of arginine for lysine, and/or the replacement of amino acid(s) with lysine comprises the replacement of at least one arginine with lysine.
3. An LDV of claim 1 which contains 1-6 lysine residues.
4. An LDV of claim 3 which contains 1-4 lysine residues.
5. A lymphokine LDV of claim 1, wherein the lymphokine is IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, G-CSF, M-CSF, GM-CSF or EPO.
6. A DNA molecule encoding an LDV of claims 1-5.
7. A procaryotic or eucaryotic host cell containing a DNA molecule of claim 6 in operable association with a transcription control sequence permitting expression of the DNA molecule and production of the LDV.
8. A method for producing an LDV of a protein or polypeptide having biological activity characterized by the deletion of, or amino acid substitution for, at least one lysine residue; and/or the insertion of a lysine residue within the polypeptide sequence and/or the replacement of a different amino acid within the polypeptide sequence with a lysine residue, which method comprises culturing a procaryotic or eucaryotic host cell

containing and capable of expressing a DNA molecule encoding the LDV under suitable conditions permitting production of the LDV.

9. A modified LDV, wherein each lysine residue of the polypeptide sequence of the LDV is linked to a hydrophilic moiety by covalent linkage of the ϵ -amino group of each lysine residue present within the polypeptide sequence of the LDV to a hydrophilic moiety selected from the group consisting of a polyalkylene glycol and succinic anhydride.

10. A method for producing a homogeneous composition containing a modified LDV of claim 9 of the formula:



wherein "-----" represents the polypeptide backbone of the LDV, "Lys" represents a lysine residue within the polypeptide sequence, "Y" represents a hydrophilic moiety covalently linked to the ϵ -amino group of the lysine residue(s); and "n" is an integer, the method comprising reacting the LDV with an amine reactive compound selected from the group consisting of a polyalkylene glycol and succinic anhydride under suitable conditions and in sufficient amounts permitting the covalent attachment of the hydrophilic moiety to each lysine residue present in the polypeptide backbone of the LDV.

11. A method of claim 10 which further comprises recovering and purifying the modified LDV so produced.

12. A modified LDV produced according to the method of claim 11.

13. A pharmaceutical composition containing a therapeutically effective amount of a modified LDV of claim 12 and a pharmaceutically acceptable carrier.

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FIG. 1

5' TCTCTTTAATCACTACTCACAGTAACCTCAACTCCTGCCACA
 -20 -10
 Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu
 ATG TAC AGG ATG CAA CTC CTG TCT TGC ATT GCA CTA AGT CTT GCA CTT
 50
 Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu
 GTC ACA AAC AGT GCA CCT ACT TCA AGT TCT ACA AAG AAA ACA CAG CTA
 100
 Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Ser Asn Gly Ile
 CAA CTG GAG CAT TTA CTT CTG GAT TTA CAG ATG ATT TCG AAT GGA ATT
 150
 Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe
 AAT AAT TAC AAG AAT CCC AAA CTC ACC AGG ATG CTC ACA TTT AAG TTT
 200
 Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu
 TAC ATG CCC AAC AAG GCC ACA GAA CTG AAA CAT CTT CAG TGT CTA GAA
 250
 Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys
 GAA GAA CTC AAA -CCT CTG GAG GAA GTG CTA AAT TTA GCT CAA AGC AAA
 350
 Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile
 AAC TTT CAC TTA AGA CCC AGG GAC TTA ATC AGC AAT ATC AAC GTA ATA
 350
 Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala
 GTT CTG GAA CTA AAG GGA TCT GAA ACA ACA TTC ATG TGT GAA TAT GCT
 400
 Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe
 GAT GAG ACA GCA ACC ATT GTA GAA TTT CTG AAC AGA TGG ATT ACC TTT
 450
 Cys Gln Ser Ile Ile Ser Thr Leu Thr
 TGT CAA AGC ATC ATC TCA ACA CTG ACT TGA TAA TTAAGTGCTTCCCACTTAAAA
 500
 CATATCAGGCCTTCTATTTATTTAAATATTTAAATTTTATATTTATTGTTGAATGTATGGTTT
 GCTACCTATTGTAACCTATTATTCTTAATCTTAAACTATAAATATGGATCTTTTATGATTCTT
 TTFGTAAGCCCTAGGGGCTCTAAAATGGTTTCACTTATTTATCCCAAATATTTATTATTATG
 TTGAATGTTAAATATAGTATCTATGTAGATTGGTTAGTAAACTATTTAATAAATTTGATAAA
 TATAAAAA

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FIG. 2

9 24 39 54
 GATCCAAAC ATG AGC OGC CTG CCC GTC CTG CTC CTG CTC CAA CTC CTG GTC OGC
 MET Ser Arg Leu Pro Val Leu Leu Leu Leu Gln Leu Leu Val Arg

(1)
 69 84 [C] 99
 CCC GGA CTC CAA GCT CCC ATG ACC CAG ACA ACG TCC TTG AAG ACA AGC TGG GTT
 Pro Gly Leu Gln Ala Pro MET Thr Gln Thr Thr Ser Leu Lys Thr Ser Trp Val

114 129 144 159
 AAC TGC TCT AAC ATG ATC GAT GAA ATT ATA ACA CAC TTA AAG CAG CCA CCT TTG
 Asn Cys Ser Asn MET Ile Asp Glu Ile Ile Thr His Leu Lys Gln Pro Pro Leu
 50

174 189 204
 CCT TTG CTG GAC TTC AAC AAC CTC AAT GGG GAA GAC CAA GAC ATT CTG ATG GAA
 Pro Leu Leu Asp Phe Asn Asn Leu Asn Gly Glu Asp Gln Asp Ile Leu MET Glu

219 234 249 264
 AAT AAC CTT OGA AGG CCA AAC CTG GAG GCA TTC AAC AGG GCT GTC AAG AGT TTA
 Asn Asn Leu Arg Arg Pro Asn Leu Glu Ala Phe Asn Arg Ala Val Lys Ser Leu

279 294 309 324
 CAG AAC GCA TCA GCA ATT GAG AGC ATT CTT AAA AAT CTC CTG CCA TGT CTG CCC
 Gln Asn Ala Ser Ala Ile Glu Ser Ile Leu Lys Asn Leu Leu Pro Cys Leu Pro

339 354 369
 CTG GCC ACG GCC GCA CCC ACG OGA CAT CCA ATC CAT ATC AAG GAC GGT GAC TGG
 Leu Ala Thr Ala Ala Pro Thr Arg His Pro Ile His Ile Lys Asp Gly Asp Trp
 MET

384 399 414 429
 AAT GAA TTC OGG AGG AAA CTG ACG TTC TAT CTG AAA ACC CTT GAG AAT GCG CAG
 Asn Glu Phe Arg Arg Lys Leu Thr Phe Tyr Leu Lys Thr Leu Glu Asn Ala Gln

444 459 475 485 495
 GCT CAA CAG ACG ACT TTG AGC CTC GCG ATC TTT T-AGTCCAAACG TCCAGCTGGT TCTCTGGGCC
 Ala Gln Gln Thr Thr Leu Ser Leu Ala Ile Phe

147

505 515 525 535 545 555 565
 TTCTCACCAC AGCGCCTCGG GACATCAAAA ACAGCAGAAC TTCTGAAACC TCTGGGTCAT CTCTCACACA

575 585 595 605 615 625 635
 TTCCAGGACC AGAAGCATT T CACCTTTTCC TGGCGCATCA GATGAATTGT TAATTATCTA ATTTCTGAAA

645 655 665
 TGTCAGCTC CCATTGGGC TTGTGGGTT GGTTCCTCA

10				20				30				40				50			
GAATTCCGGG				AAOGAAAGAG				AAGCTCTATC				TCCOCTCCAG				GAGCCACAGCT			
																ATG AAC TCC TTC			
																MET Asn Ser Phe			
65				80				95				110							
TCC	ACA	AGC	GCC	TTC	GGT	CCA	GTT	GCC	TTC	TCC	CTG	GGG	CTG	CTC	CTG	GTC	TTC		
Ser	Thr	Ser	Ala	Phe	Gly	Pro	Val	Ala	Phe	Ser	Leu	Gly	Leu	Leu	Leu	Val	Leu		
125				(1)				140				155				170			
CCT	GCT	GCC	TTC	CCT	GCC	CCA	GTA	CCC	CCA	GGA	GAA	GAT	TCC	AAA	GAT	GTA	GCC		
Pro	Ala	Ala	Phe	Pro	Ala	Pro	Val	Pro	Pro	Gly	Glu	Asp	Ser	Lys	Asp	Val	Ala		
185				200				215											
GCC	CCA	CAC	AGA	CAG	CCA	CTC	ACC	TCT	TCA	GAA	CGA	ATT	GAC	AAA	CAA	ATT	CGG		
Ala	Pro	His	Arg	Gln	Pro	Leu	Thr	Ser	Ser	Glu	Arg	Ile	Asp	Lys	Gln	Ile	Arg		
230				245				260				275							
TAC	ATC	CTC	GAC	GGC	ATC	TCA	GCC	CTG	AGA	AAG	GAG	ACA	TGT	AAC	AAG	AGT	AAC		
Tyr	Ile	Leu	Asp	Gly	Ile	Ser	Ala	Leu	Arg	Lys	Glu	Thr	Cys	Asn	Lys	Ser	Asn		
290				305				320											
ATG	TGT	GAA	AGC	AGC	AAA	GAG	GCA	CTG	GCA	GAA	AAC	AAC	CTG	AAC	CTT	CCA	AAG		
MET	Cys	Glu	Ser	Ser	Lys	Glu	Ala	Leu	Ala	Glu	Asn	Asn	Leu	Asn	Leu	Pro	Lys		
335				350				365				380							
ATG	GCT	GAA	AAA	GAT	GGA	TGC	TTC	CAA	TCT	GGA	TTC	AAT	GAG	GAG	ACT	TGC	CTG		
MET	Ala	Glu	Lys	Asp	Gly	Cys	Phe	Gln	Ser	Gly	Phe	Asn	Glu	Glu	Thr	Cys	Leu		
395				410				425				440							
GTG	AAA	ATC	ATC	ACT	GGT	CTT	TTG	GAG	TTT	GAG	GTA	TAC	CTA	GAG	TAC	CTC	CAG		
Val	Lys	Ile	Ile	Thr	Gly	Leu	Leu	Glu	Phe	Glu	Val	Tyr	Leu	Glu	Tyr	Leu	Gln		
455				470				485											
AAC	AGA	TTT	GAG	AGT	AGT	GAG	GAA	CAA	GCC	AGA	GCT	GTG	CAG	ATG	AGT	ACA	AAA		
Asn	Arg	Phe	Glu	Ser	Ser	Glu	Glu	Gln	Ala	Arg	Ala	Val	Gln	MET	Ser	Thr	Lys		
500				515				530				545							
GTC	CTG	ATC	CAG	TTC	CTG	CAG	AAA	AAG	GCA	AAG	AAT	CTA	GAT	GCA	ATA	ACC	ACC		
Val	Leu	Ile	Gln	Phe	Leu	Gln	Lys	Lys	Ala	Lys	Asn	Leu	Asp	Ala	Ile	Thr	Thr		
560				575				590											
CCT	GAC	CCA	ACC	ACA	AAT	GCC	AGC	CTG	CTG	ACG	AAG	CTG	CAG	GCA	CAG	AAC	CAG		
Pro	Asp	Pro	Thr	Thr	Asn	Ala	Ser	Leu	Leu	Thr	Lys	Leu	Gln	Ala	Gln	Asn	Gln		
605				620				635				650							
TGG	CTG	CAG	GAC	ATG	ACA	ACT	CAT	CTC	ATT	CTG	CGC	AGC	TTT	AAG	GAG	TTC	CTG		
Trp	Leu	Gln	Asp	MET	Thr	Thr	His	Leu	Ile	Leu	Arg	Ser	Phe	Lys	Glu	Phe	Leu		

FIG. 3 (continued)

665 680 696 706 716
CAG TCC AGC CIG AGG GCT CTT OGG CAA ATG TAGCATGGGC ACCTCAGATT GTTGTGTGTA
Gln Ser Ser Leu Arg Ala Leu Arg Gln MET

726 736 746 756 766 776 786
ATGGGCATTC CTTCTTCIGG TCAGAAACCT GTCCACTGGG CACAGAACTT ATGTTGTTCT CTATGGAGAA

796 806 816 826 836 846 856
CTAAAAGTAT GAGCGTTAGG ACACTATTTT AATTATTTTT AATTATTTAA TATTTAAATA TGTAAGCTG

866 876 886 896 906 916 926
AGTTAATTTA TGTAAGTCAT ATTTATATTT TTAAGAAGTA CCACTTGAAA CATTTTATGT ATTAGTTTTG

936 946 956 966 976 986 996
AAATAATAAT GGAAAGTGGC TATGCAGTTT GAATATCCTT TGTTTCAGAG CCAGATCATT TCTTGGAAG

1006 1016 1026 1036 1046 1056 1066
TGTAGGCTTA CCTCAAATAA ATGGCTAACT TATACATATT TTAAAGAAA TATTATATT GTATTATAT

1076 1086 1096 1106 1116 1126 1136
AATGTATAAA TGGTTTTTAT ACCAATAAAT GGCATTTTAA AAAATTCAAA AAAAAAAAAA AAAAAAGAA

TTC

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FIG. 4

1	ACC	CCC	CTG	GGC	CCT	GCC	AGC	TCC	CTG	CCC	CAG	AGC	TTC	CTG	CTC	10
	Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	
					20											30
	AAG	TGC	TTA	GAG	CAA	GTG	AGG	AAG	ATC	CAG	GGC	GAT	GGC	GCA	GCG	
	Lys	Cys	Leu	Glu	Gln	Val	Arg	Lys	Ile	Gln	Gly	Asp	Gly	Ala	Ala	
										40						
	CTC	CAG	GAG	AAG	CTG	TGT	GCC	ACC	TAC	AAG	CTG	TGC	CAC	CCC	GAG	
	Leu	Gln	Glu	Lys	Leu	Cys	Ala	Thr	Tyr	Lys	Leu	Cys	His	Pro	Glu	
					50											60
	GAG	CTG	GTG	CTG	CTC	GGA	CAC	TCT	CTG	GGC	ATC	CCC	TGG	GCT	CCC	
	Glu	Leu	Val	Leu	Leu	Gly	His	Ser	Leu	Gly	Ile	Pro	Trp	Ala	Pro	
										70						
	CTG	AGC	AGC	TGC	CCC	AGC	CAG	GCC	CTG	CAG	CTG	GCA	GGC	TGC	TTG	
	Leu	Ser	Ser	Cys	Pro	Ser	Gln	Ala	Leu	Gln	Leu	Ala	Gly	Cys	Leu	
					80											90
	AGC	CAA	CTC	CAT	AGC	GGC	CTT	TTC	CTC	TAC	CAG	GGG	CTC	CTG	CAG	
	Ser	Gln	Leu	His	Ser	Gly	Leu	Phe	Leu	Tyr	Gln	Gly	Leu	Leu	Gln	
										100						
	GCC	CTG	GAA	GGG	ATC	TCC	CCC	GAG	TTG	GGT	CCC	ACC	TTG	GAC	ACA	
	Ala	Leu	Glu	Gly	Ile	Ser	Pro	Glu	Leu	Gly	Pro	Thr	Leu	Asp	Thr	
					110											120
	CTG	CAG	CTG	GAC	GTC	GCC	GAC	TTT	GCC	ACC	ACC	ATC	TGG	CAG	CAG	
	Leu	Gln	Leu	Asp	Val	Ala	Asp	Phe	Ala	Thr	Thr	Ile	Trp	Gln	Gln	
										130						
	ATG	GAA	GAA	CTG	GGA	ATG	GCC	CCT	GCC	CTG	CAG	CCC	ACC	CAG	GGT	
	Met	Glu	Glu	Leu	Gly	Met	Ala	Pro	Ala	Leu	Gln	Pro	Thr	Gln	Gly	
					140											150
	GCC	ATG	CCG	GCC	TTC	GCC	TCT	GCT	TTC	CAG	CGC	CGG	GCA	GGA	GGG	
	Ala	Met	Pro	Ala	Phe	Ala	Ser	Ala	Phe	Gln	Arg	Arg	Ala	Gly	Gly	
										160						
	GTC	CTG	GTT	GCC	TCC	CAT	CTG	CAG	AGC	TTC	CTG	GAG	GTG	TCG	TAC	
	Val	Leu	Val	Ala	Ser	His	Leu	Gln	Ser	Phe	Leu	Glu	Val	Ser	Tyr	
					170					174						
	CGC	GTT	CTA	CGC	CAC	CTT	GCC	CAG	CCC	T						
	Arg	Val	Leu	Arg	His	Leu	Ala	Gln	Pro							

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FIG. 5A

1. 5' AATTGCGCGCCACCATGTACAGGATGCAACTCCTGTCTTGCCATGGCACTAAGTCTTGCA
2. 5' GACAAGTGCAAGACTTAGTGCAATGCAAGACAGGAGTTGCATCCTGTACATGGTGGCGGCG
3. 5' CTTGTCACAAAACAGTGCACCTACTAGCTCGAGTACAAGAAAGAACACAGCTACAACTGGAG
4. 5' TAAATGCTCCAGTTGTAGCTGTGTCTTCTTGTACTCGAGCTAGTAGGTGCACACTGTTGT
5. 5' CATTTACTTCTGGATCTGCAGATGATTTTCGAATGGAATTAAATTAACAGAAATCCTTAGG
6. 5' GGTGAGCCTAGGATTTCTGTAAATTATTAATTCCTCGAAATCATCTGCAGATCCAGAAAG
7. 5' CTCACCAGGATGCTCATTTCAGATTCTACATGCCCCAGAAAGGCCACAGAACTGAGACAT
8. 5' CTGAAGATGTCTCAGTTCTGTGGCTCTTCTGGGCATGTAGAATCTGAATGTGAGCATCCT
9. 5' CTTCAGTGTCTAGAAGAAAGAACTCAGACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
10.5' GTTCTTGCTTTGAGCTAAATTTAGCACTTCTCTCCAGAGGTCTGAGTTCTTCTCTAGACA
11.5' AAGAACTTTCACTTAAGACCCCGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAA
12.5' TCTTAGTTCCAGAACTATTACGTTGATATTGCTGATTAAAGTCCCGGGGTCTTAAGTGAA
13.5' CTAAGAGGATCCGAAAACAACATTCAATGTGTGAATATGCTGATGAGACAGCAACCATTTGTA
14.5' GAATTCTACAATGGTTGCTGTCTCATCAGCATATTACACATGAATGTTTTCGGATCC
15.5' GAATTCCTGAACAGATGGATTACCTTTGCTCAAAGCATCATCTCAACACTGACTTGATAAC
16.5' TCGAGTTATCAAGTCAGTGTGTGAGATGATGCTTTGAGCAAGGTAATCCATCTGTTTCAG

FIG. 5B

1. 5' AATTGCGCGCCACCATGTACAGGATGCAACTCCTGTCTTGTGCACTAAGTCTTGCA
2. 5' GACAAAGTGCAAGACTTAGTGCAATGCAAGACAGGAGTTGCATCCTGTACATGGTGGCGGCG
3. 5' CTTGTCAAAAACAGTGCACCTACTAGCTCGAGTACAAGAAGAACACAGCTACAACTGGAG
4. 5' TAAATGCTCCAGTTGTAGCTGTGTCTTCTTGTACTCGAGCTAGTAGGTGCACTGTTTGT
5. 5' CATTTACTTCTGGATCTGCAGATGATTTCCGAATGGAATTAATAATTACAGAAATCCTAGG
6. 5' GGTGAGCCTAGGATTTCTGTAAATTATTAAATTCCTCCAAATCATCTGCAGATCCAGAAG
7. 5' CTCACCAGGATGCTCACATTCAGATTCTACATGCCCAAGGCCACAGAACTGAGACAT
8. 5' CTGAAAGATGTCTCAGTTCTGTGGCTCTTCTGTGGCATGTAGAAATCTGAATGTGAGCATCCT
9. 5' CTTCAGTGTCTAGAAGAAAGAACTCAGACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
10. 5' GTTCTTGCTTTGAGCTAAATTTAGCACTTCCCTCCAGAGGTCTGAGTTCTTCTTCTAGACA
11. 5' AAGAACTTTCACTTAAGACCCCGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAA
12. 5' TCCTTAGTTCAGAACTATTACGTTGATATTGCTGATTAAAGTCCCGGGTCTTAAGTGA
13. 5' CTAAGAGGATCCGAAACAACATTCATGTGTGAATATGCTGATGAGACAGCAACCATTTGTA
14. 5' GAATTCTACAATGGTTGCTGTCTCATCAGCATATTTCACACATGAATGTTGTTTCGGATCC
15. 5' GAATTCCTGAACAGATGGATTACCTTTTGTCAAAGCATCATCTCAACACACTGACTTGATAAC
16. 5' TCGAGTTATCAAGTCAGTGTGTGAGATGATGCTTTTGAGCAAAGGTAATCCATCTGTTTCAG

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/04633

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC4: C 07 K 13/00, C 21N 15/00, A61 K 37/02, 47/00, 45/02																							
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched †</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">IPC4</td> <td style="padding: 5px;">A 61 K; C 07 K; C 12 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *</div>			Classification System	Classification Symbols	IPC4	A 61 K; C 07 K; C 12 N																	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT * <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category *</th> <th style="border-bottom: 1px solid black;">Citation of Document, †† with indication, where appropriate, of the relevant passages ‡</th> <th style="border-bottom: 1px solid black;">Relevant to Claim No. ‡</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="padding: 5px;">EP, A1, 0163529 (NOVA INDUSTRI A/S) 4 December 1985, See Abstract, Claims</td> <td style="text-align: center; vertical-align: top;">1-4,6-8</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="text-align: center; vertical-align: top;">--</td> <td style="text-align: center; vertical-align: top;">9-13</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="padding: 5px;">EP, A2, 0237967 (OTSUKA PHARMACEUTICAL CO., LTD.) 23 September 1987, see claims 1,2,15,19,20</td> <td style="text-align: center; vertical-align: top;">1,3-8</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="text-align: center; vertical-align: top;">--</td> <td style="text-align: center; vertical-align: top;">9-13</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="padding: 5px;">EP, A1, 0194006 (IMERIAL INDUSTRIES PLC) 10 September 1986, see See Claims 1,12,14,14,15,16,18,20</td> <td style="text-align: center; vertical-align: top;">1,3-8</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="text-align: center; vertical-align: top;">--</td> <td style="text-align: center; vertical-align: top;">9-13</td> </tr> </tbody> </table>			Category *	Citation of Document, †† with indication, where appropriate, of the relevant passages ‡	Relevant to Claim No. ‡	X	EP, A1, 0163529 (NOVA INDUSTRI A/S) 4 December 1985, See Abstract, Claims	1-4,6-8	Y	--	9-13	X	EP, A2, 0237967 (OTSUKA PHARMACEUTICAL CO., LTD.) 23 September 1987, see claims 1,2,15,19,20	1,3-8	Y	--	9-13	X	EP, A1, 0194006 (IMERIAL INDUSTRIES PLC) 10 September 1986, see See Claims 1,12,14,14,15,16,18,20	1,3-8	Y	--	9-13
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<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>* Special categories of cited documents: ††</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>																							
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 23rd March 1989 </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report 13. 04. 89 </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer P.C.G. VAN DER PUTTEN </td> </tr> </table>			Date of the Actual Completion of the International Search 23rd March 1989	Date of Mailing of this International Search Report 13. 04. 89	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer P.C.G. VAN DER PUTTEN																	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<p>Russian Chemical Reviews, Vol. 49, No. 3, 1980 I.N. Topchièva: "Biochemical Applications of Poly(Ethylene Glycol) ", see page 260 - page 271 See page 266, column 1, line 58 - column 2, line 18</p> <p>-- -----</p>	9-13

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 88/04633**

SA 26054

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/01/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0163529	04/12/85	JP-A- 61001389	07/01/86
		AU-D- 43092/85	05/12/85

EP-A2- 0237967	23/09/87	EP-A- 0237073	16/09/87
		JP-A- 63152398	24/06/88
		JP-A- 63164899	08/07/88

EP-A1- 0194006	10/09/86	JP-A- 61275300	05/12/86
